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Comparison of the Canine and Human Acid β-Galactosidase Gene

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Several canine cDNA libraries were screened with human β-galactosidase cDNA as probe. Seven positive clones were isolated and sequenced yielding a partial (2060 bp) canine β-galactosidase cDNA with 86% identity to the human β-galactosidase cDNA. Preliminary analysis of a canine genomic library indicated conservation of exon number and size. Analysis by Northern blotting disclosed a single mRNA of 2.4 kb in fibroblasts and liver from normal dogs and dogs affected with GM1 gangliosidosis. Although incomplete, these results indicate canine GM1 gangliosidosis is a suitable animal model of the human disease and should further efforts to devise a gene therapy strategy for its treatment. © 1996 Wiley-Liss, Inc.

KEY WORDS: canine, β-galactosidase, GM1 gangliosidosis, animal model, gene therapy

INTRODUCTION

Animal models, the occurrence of genetic diseases in animals similar to ones known to exist in humans, are valuable and essential research tools. The knowledge gained from animal models has increased our understanding of animal and human diseases and has added to and modified conceptions of the normal mechanisms that are physiologically operative. One of the greatest potential benefits of an available animal model is its use in the evaluation of therapeutic approaches.

The sophisticated techniques that are now becoming available make it possible to pursue strategies for implementing gene therapy as a viable treatment for previously untreatable "inborn errors of disease", such as the lysosomal storage diseases. This therapeutic approach may be realized when the functional gene is properly targeted to the appropriate tissue and properly expressed and regulated. Prior to human application, these potential treatments must be established to be efficacious and nontoxic in experimental animals. Fortunately, numerous animal models of lysosomal storage diseases have been identified in several mammalian species [Desnick et al., 1982].

GM1 gangliosidosis is a fatal lysosomal storage disease that is caused by a deficiency of lysosomal acid β-galactosidase [for review see O'Brien, 1989]. It has been reported in cattle [Donnelly and Sheahan, 1981], cats [Blakemore, 1972], dogs [Read et al., 1976], humans [Okada and O'Brien, 1968], and sheep [Ahern-Rindell et al., 1988]. Canine GM1 gangliosidosis has been observed in several breeds including: mixed breed Beagle [Read et al., 1976; Rodriguez et al., 1982], English Springer Spaniel [Alroy et al., 1985], and Portuguese Water Dog [Sauders et al., 1988; Shell et al., 1989]. The canine disease resembles the human disease genetically, clinically, biochemically, and pathologically.

Transmission of the disease is through an autosomal recessive pattern of inheritance [Read et al., 1976]. Clinical symptoms vary somewhat among cases but all show progressive neurological impairment with progressive ataxia and tremors. Cells and tissues of affected dogs show greatly reduced levels of acid β-galactosidase activity [Read et al., 1976; Rodriguez et al., 1982]. Levels of GM1 ganglioside and its asialo derivative are elevated in brain and galactosyl-containing oligosaccharides accumulate in tissues and are excreted at high levels in urine [Warner et al., 1983].

The pathologic presentation is characteristic of lysosomal storage diseases with no gross lesions and no significant neuronal loss observed at necropsy. However, microscopic evaluation discloses enlarged neurons that are vacuolated with foamy and/or granular cytoplasm. Ultrastructurally these neuronal inclusions consist of concentrically wound membranes arranged in whorl patterns or lamellar stacks [Read et al., 1976; Rodriguez et al., 1982].

The resemblance between canine and human GM1 gangliosidosis suggests that similarities exist at the molecular level as well. This prompted our cloning the canine β-galactosidase gene using human β-galactosidase cDNA as a probe. We have found a high level of identity

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between the canine and human acid β-galactosidase gene supporting the use of canine GM1 gangliosidosis as an animal model of the human disorder.

MATERIALS AND METHODS

cDNA Library Screening

The following four canine cDNA libraries were screened: λgt10 kidney library (Clontech, Palo Alto, CA), λ ZAP® II testis library (Stratagene, La Jolla, CA), a λ ZAP® II muscle library (Stratagene) and a λ ZAP® II pancreas library (Stratagene). Approximately 3×10^6 plaques were screened from the pancreas library alone after plating on *E. coli* XL1-Blue cells. The phage DNA was transferred to nitrocellulose and fixed by heating at 80°C under vacuum for 2 hr. The DNA-fixed filters were prehybridized at 37°C for at least 1 hr in a solution containing 5× SSPE (sodium chloride/sodium phosphate), 50% formamide, 0.1% SDS (sodium dodecyl sulfate), 100 μg/ml of denatured salmon sperm DNA, and 3× Denhardt's solution (Ficoll/polyvinyl pyrrolidone/bovine serum albumin).

The filters were hybridized at 37°C overnight in the prehybridization solution with the addition of ^{32}P-labeled probe. The probe was prepared from human β-galactosidase cDNA, cloned previously in the laboratory of J.S. O'Brien [Yamamoto et al., 1990], and labeled with α-^{32}P dCTP using US Biochemical's (Cleveland, OH) Random-Primed Labeling Kit.

The filters were washed two times at room temperature for 5 min each with a solution of 1× SSPE and 0.1% SDS; one time at 45°C and one time at 60°C, for 15 min each with a solution of 0.1× SSPE and 0.1% SDS; and finally one time at room temperature for 5 min with 0.05× SSPE. The wet filters were placed between plastic sheets and exposed to Kodak X-Omat film at −70°C with an intensifying screen. Seven positive clones were isolated after 5 rounds of screening and used for nucleotide sequence analysis.

Plasmid Excision and Sequencing

pBluescript SK⁻ plasmids containing inserts from the positive clones were obtained by in vivo excision from the λ ZAP® II vector using the interference resistant helper phage R408 (Stratagene). The positive clone phage stocks were combined with XL1-Blue cells and R408 helper phage and incubated at 37°C for 15 min. A yeast extract broth was added to the cells and phage and incubated at 37°C for 3 hr with shaking. This was followed with a 70°C incubation for 20 min and a 5-min centrifugation at 4,000 × g.

The supernatant containing the excised pBluescript plasmid was plated out with XL1-Blue cells on LB/Ampicillin plates and incubated overnight at 37°C. Colonies picked from the plates contained pBluescript double-stranded plasmid with insert which were then used for sequencing with modified T7-DNA polymerase (Sequenase, US Biochemicals) according to instructions for double-stranded template.

Genomic Library Screening

A canine spleen genomic library constructed in the Lambda DASH™ vector (Stratagene) containing inserts ranging from 9 to 22 kb, was screened after plating on SRB (Stratagene) cells. The phage DNA was transferred to nitrocellulose and fixed by heating at 80°C under vacuum for 2 hr. The prehybridization (2 hr at 42°C), hybridization (overnight at 42°C) and wash procedures (30 min at 65°C, three times) were performed according to manufacturer's instructions for a double-stranded probe.

The probe was prepared from canine β-galactosidase cDNA and labeled with γ-^{32}P dCTP using US Biochemical's Random-Primed Labeling Kit. The wet filters were placed between plastic sheets and exposed to Kodak X-Omat film at −70°C with an intensifying screen.

RNA Isolation and Northern Blot Preparation

Poly(A) RNA was prepared from canine fibroblasts and liver tissue using the Fast-Track RNA isolation kit (Invitrogen, San Diego, CA) as recommended by the manufacturer. RNA was electrophoretically separated on a 1% formaldehyde/agarose gel [Maniatis et al., 1982] and blotted onto a nylon membrane (Schleicher & Schuell, Keene). Canine cDNA clones were used as probes for hybridization by labeling with the Random-Primed DNA Labeling kit (US Biochemicals), with Klenow enzyme and α-^{32}P dCTP (Amersham, Arlington Heights). Hybridization was performed in 50% formamide/5× SSC at 55°C overnight according to the manufacturer's specifications.

The nylon membrane was washed at room temperature in 6× SSC/0.1% SDS twice for 15 min each, and twice in 1× SSC/0.1% SDS for 15 min each at 42°C. The washed membrane was exposed to Kodak X-Omat film for 24 hr at −70°C with an intensifying screen.

Assay of β-Galactosidase Activity

Fibroblast cells and tissue samples from Portuguese Water Dogs (kindly provided by Jane Harding of Cutwater Kennels), were assayed for β-galactosidase activity using 4-methylumbelliferyl β-D-galactoside as substrate [Rodriguez et al., 1982]. Determination of β-galactosidase activity was based on a standard curve of 4-methylumbelliferone (4-MU). Protein concentration was determined by the method of Bradford [1976] with bovine serum albumin as the standard. Enzymatic activity was calculated as nanomoles of 4-MU liberated per hr per mg of protein (nmol 4-MU/hr/mg protein).

Umbilical cords from newborn pups were assayed initially to genotypically identify them as normal, carriers, or GM1 affected. Skin biopsies were then performed and the skin samples sent directly to the Mutant Cell Line Repository (Camden, New Jersey) to establish fibroblast cell lines. Two canine fibroblast cell lines were successfully established from the GM1 gangliosidosis-affected Portuguese Water Dogs (GM11473 and GM11474).

RESULTS

cDNA Library Screening

Screening of the canine cDNA libraries with human cDNA probes produced several positive clones. Sequencing of these clones yielded approximately 2,000 base pairs of composite sequence, plus the termination sequence and polyadenylation signal. A comparison between the human and canine cDNA sequences thus far

indicates approximately 86% identity at the nucleotide level and about 81% identity at the amino acid level with approximately half of the amino acid substitutions being conservative (Fig. 1). The partial sequence of the canine β-galactosidase cDNA has been submitted to GenBank.

Genomic Library Screening

Screening of a canine spleen genomic library produced six positive clones when probed with the largest isolated canine cDNA clone of 1,900 bases. Initial analysis of these genomic clones yielded 225 additional bases of sequence at the 5′ end of the gene. We estimate that there are still approximately 75 unsequenced bases at the 5′ end of the canine cDNA based on a comparison with the human β-galactosidase cDNA sequence (Fig. 1).

Preliminary analysis of the genomic clones yielded sequence information pertaining to 21 of the estimated 30 canine exon/intron boundaries. Exon number and size appears to be conserved between human and dog (Table I).

Northern Blot Analysis

We isolated mRNA from dog tissues and cells on several occasions, one time from dog liver and on two separate occasions from dog fibroblast cultures. Each time a band of approximately 2.4 kb in size was obtained when probed with the canine cDNA clone of 1,900 bases (Fig. 2). Each of the mRNA isolations utilized tissue or cells from control dogs and dogs affected with GM1 gangliosidosis. For all three isolations, the mRNA obtained from the GM1 affected dogs was similar in size to the mRNA from control dogs.

β-Galactosidase Activity

The β-galactosidase activity in umbilical cords from newborn Portuguese Water Dog pups was used to identify the dogs as normal, homozygous dominant (600–1,500 nmol 4-MU/hr/mg prot); heterozygous carriers (150–300 nmol 4-MU/hr/mg prot); and GM1 affected homozygous recessive (3–30 nmol 4-MU/hr/mg protein).

DISCUSSION

We have successfully used human cDNA fragments encoding β-galactosidase to isolate partial canine β-galactosidase cDNAs from several cDNA libraries. The largest cDNA fragment consisted of 2,060 base pairs, contained a poly(A) tail but was lacking approximately 300 base pairs from the 5′ end when compared to human β-galactosidase cDNA. An 86% identity at the nucleotide level between canine and human β-galactosidase cDNA exceeds expectations of 60–80% identity predicted from a comparison of chymotryptic and tryptic peptide maps [Hubert and O'Brien, 1983].

After screening four cDNA libraries and sequencing a dozen clones, no clones were found to extend 5′ more so than approximately 300 bp from the predicted initiation codon. We believe that there may be some secondary structure in the 5′ region which interfered with reverse transcription preventing synthesis of a full-length cDNA. This could explain why half of the isolated clones were truncated at the same position.

We attempted polymerase chain reaction (PCR) amplification using these cDNA libraries as templates with a 3′ primer based on canine β-galactosidase sequence from the 5′-most end of our clones and a 5′ primer whose sequence was based on the human 5′ β-galactosidase sequence. No PCR product was obtained from these libraries. Perhaps these libraries did not contain the template, i.e., 5′ canine β-galactosidase sequence, or the 5′ primer did not anneal to the template because of sequence differences.

We also tried unsuccessfully to obtain the 5′ sequence of canine β-galactosidase by using a genomic library as template for PCR. This may have been unsuccessful because of inadequate homology between the canine and human cDNA sequence in this 5′ region. The first 23 amino acids of the human cDNA serves as a signal sequence [Yamamoto et al., 1990]. It has little homology to the mouse signal sequence, <50% [Nanba and Suzuki, 1991] and most likely, little homology to the canine signal sequence as well.

Based on information we have obtained from the literature regarding the human and mouse β-galactosidase genes [Morreau et al., 1991; Nanba and Suzuki, 1991], the signal sequence is a part of exon I. The first exon/intron boundary is just after the signal sequence cleavage site, just before amino acid 26 in human β-galactosidase cDNA. The 5′ end of the canine cDNA sequence we have identified appears to coincide with the beginning of exon II, meaning that the 5′ canine bases that are missing probably make up part of exon I of the canine gene.

We estimate that after identifying the additional 25 amino acids coded for at the 5′ end of the dog β-galactosidase gene, the total will come to 667. This is 10 fewer than that for which the human gene codes, 677 [Morreau et al., 1991] and 20 more than that for which the mouse gene codes, 647 [Nanba and Suzuki, 1991]. These differences are found near the end of the open reading frame at the carboxy terminus.

The site of the human gene that codes for the proposed proteolytic cleavage, amino acid 530 [Yamamoto et al., 1990], could also serve as the site for cleavage in the dog as determined by a Kyte and Doolittle [1982] hydropathy plot which shows very close alignment between the human and dog amino acid sequences (data not shown).

Glycosylation sites (Asn-x-Thr or Ser) of which there are 6 in the dog β-galactosidase, are the same as in human with the exception that human β-galactosidase has an additional site at amino acid 542 [Yamamoto et al., 1990]. The mouse β-galactosidase by comparison has 8 sites; 5 of them are the same as 5 of human and dog [Nanba and Suzuki, 1991].

Cysteine residues in dog β-galactosidase are the same 7 found in mouse and human β-galactosidase with the only exception being the human enzyme, which has an additional cysteine moiety at amino acid 258 [Yamamoto et al., 1990; Nanba and Suzuki, 1991].

As described above, we began screening a dog genomic library in an attempt to find the 5′ sequence of the canine cDNA. During this process, we identified several of the dog exon/intron boundaries. We believe

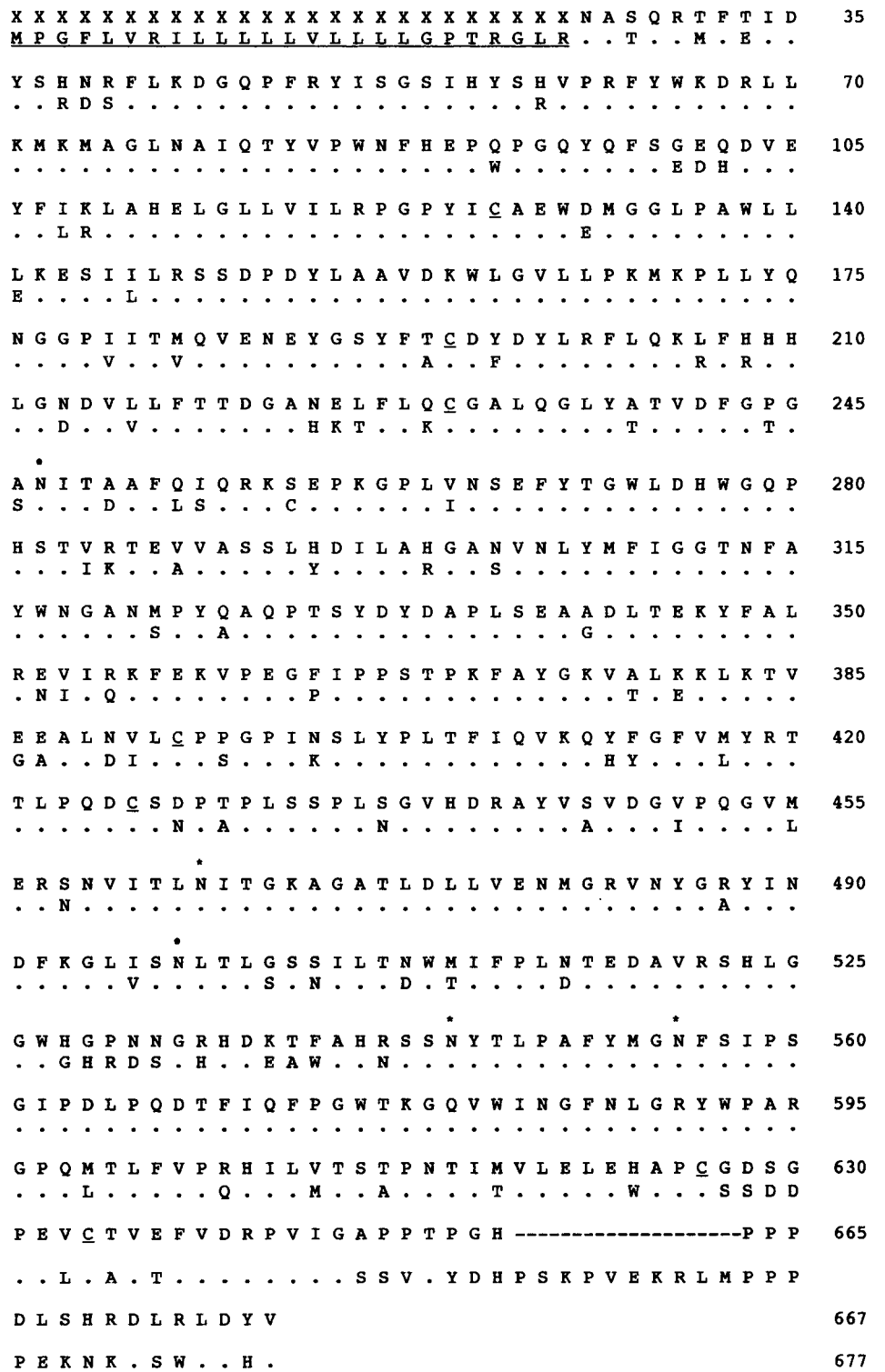

Fig. 1. A comparison of the deduced canine and human β-galactosidase amino acid sequence. Top line depicts canine, and bottom line depicts human β-galactosidase amino acid sequence with only differences indicated. Underlined sequence identifies human β-galactosidase signal sequence. Absence of corresponding canine signal sequence is shown by a series of Xs. Asterisks denote potential glycosylation sites, and cysteine residues are underlined. Broken line in dog sequence suggests the location of the 10 fewer amino acids as compared to the human sequence. The designation of amino acid 1 was made to the methionine encoded by the first ATG of the human sequence.

TABLE I. Nucleotide Sequence of Exon/Intron Boundaries in the Canine β-Galactosidase Gene*

Exon	Size	5′ Splice donor	3′ Splice acceptor
I	[a]	—	tgtggtcccatccttgctctgcag/AATGCTTCC
II	170	CATCCAGAC/gtaagtaagagggcgctgggctct	aagggactgtgtgtgtcttggcag/GTACGTGCC
III	151	TGGGACATG/gtgagtatggctgggcaggctcta	tttcgcccaccacccccattgtag/GGAGGATTA
IV	61	CTGATCCAG/gtaagttgtacatgatgtctcgag	agttaaacctgtcttgttttgcag/ATTACCTTG
V	95[a]	ACCATGCAG/gtaacctcagacatgagcaggtgg	— /GTTGAAAAT
VI	181	TTGGACCAG/gttggtgtttgtgatcacagaaaa	tcttctcactctcataatttccag/GTGCCAACA
VII	59	GGACCATTG/gtgagaattggggtgaagtgtggg	tccatgtcctttcctcttttttag/GTGAATTCT
VIII	122	TGTGAACTT/gtgagtgctaattgctagaacaga	atatgttctcttgtttccttgtag/GTACATGTT
IX	41[a]	ATTGGAATG/ —	— /GGGCCAACA
X	113[a]	ATTCGGAAG/ —	— /TTTGAAAAA
XI	75	CTGAAGAAG/gtaagagagcaaataaacaaaggt	aatgatgttttggtttcttttcag/TTAAAGACG
XII	90[a]	GTGAAACAG/gtaggccttcaaaggtgatgcttc	— /TATTTCGGT
XIII	114[a]	GTGGATGGG/gtaagaattgtcactgagctgtgc	tcccactggtctctaaccccacag/GTGCCCCAG
XIV	132	GATTTTAAG/gtaggaccagccccactgtcagga	attgctcagtttctcctctaacag/GGCCTTATT
XV	252[a]	TGGACCAAG/ —	— /GGTCAGGTG
XVI	567[a]		

*Exon sequence is in capital letters and intron sequence is in lowercase letters.
[a]Sufficient sequence is not available to substantiate exon size. Instead, size of canine exon is estimated based on a comparison to the human β-galactosidase gene.

the dog gene probably contains 16 exons as is seen in the human and mouse genes [Morreau et al., 1991; Nanba and Suzuki, 1991]. All of the boundaries we sequenced in the dog gene coincide with those found in the human gene, making the respective exons the same size except for the last exon, XVI. The mouse exons are almost all the same size as dog and human except for exons I, XIII, and XVI.

Northern blots prepared from mRNA isolated from both control and affected GM1 gangliosidosis dogs yielded a band of 2.4 kb in size compared to human mRNA of size 2.45 kb [Yamamoto et al., 1990]. This indicates that the mutation causing GM1 gangliosidosis in Portuguese Water Dogs is likely the result of a change within the coding sequence of the dog β-galactosidase gene. Nothing conclusively can be said about quantity differences in the mRNA from normal dogs compared to GM1 affected dogs since mRNA concentrations were not directly determined.

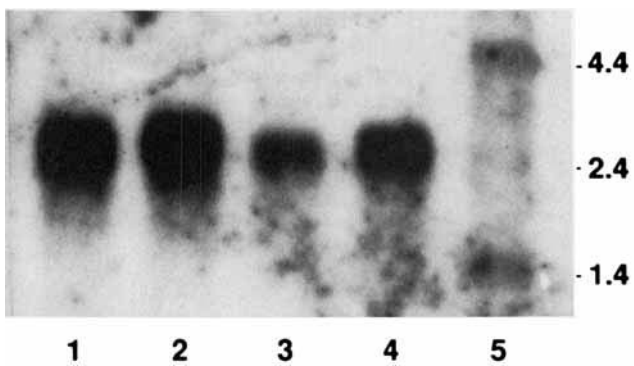

Fig. 2. Northern blot analysis of fibroblast poly(A) RNA. Samples of poly(A) RNA from normal canine fibroblasts (GM08462) are shown in **lanes 1** and **2** (different passages of the same cell line) and from fibroblasts of dogs affected with GM1 gangliosidosis are shown in **lanes 3** (GM11473) and **4** (GM11474). Size standards are shown in **lane 5** (RNA ladder, Bethesda Research Laboratories, Gaithersburg, MD 0.24–9.5 kb).

Our findings suggest a high level of homology between the human and dog β-galactosidase gene. This further supports the use of canine GM1 gangliosidosis as an animal model of the human disorder. Mutations causing GM1 gangliosidosis in Portuguese Water Dogs and English Springer Spaniels are probably not the same because of their different clinical, biochemical and histochemical profiles [Alroy et al., 1992]. The availability of the canine β-galactosidase cDNA sequence paves the way for future studies to identify the mutation(s) in the dog models of GM1 gangliosidosis and should further efforts to devise a gene therapy strategy for human GM1 gangliosidosis using an animal model.

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